

Antitumor activity of plant associated endophytic actinomycetes

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General Note



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ABSTRACT

The current analysis segregated the endophytic actinomycetes from bio-materials like cabbage (*Brassica oleracea*), tomato (*Solanum lycopersicum*) and maize (*Zea mays*). The total 40 actinomycetes were isolated using Starch casein agar. The huge anti-bacterial action found in 4 (10%) endophytes among forty endophytic actinomycete samples. They were recovered using agar streak method on Starch casein agar. The production of antimicrobial compound was performed by fermenting ISP 2 broth with 4 isolates for 15 days. After fermentation, extraction of the supernatant was carried out using solvent ethyl acetate. Analysis of 16S rDNA sequencing of those isolates showed that strain 34 belong to member of the genera *Streptomyces sampsonii*. Column & TLC Chromatography techniques were utilized to recover the anti-bacterial analogs. The HPLC-UV chromatogram of the extracts exhibited most exciting diversity of the chemical constituents. Crude extract of S34 exhibited three different peaks at retention time (RT) 1.905, 2.608 and 3.027. Investigation of tumor selectivity of the 4 endophytic actinomycete metabolites against selected Breast cancer cell lines indicated effective and promising differential cytotoxicity. Strain 15 exhibited high antitumour activity of R^2 value 0.9802. The bio-compounds were recognized, screened and analyzed through NRPS by exact amplification.

Keywords: Endophytic actinomycetes, *Streptomyces* sp, Antitumour activity, HPLC and NMR analysis

1. INTRODUCTION

Endophytic microbes exploit an unusual habitat (i.e. internal living tissues of plants), and this may enable them to possess the potential to produce bioactive compounds which can be used to design new drugs replacing those against which pathogenic strains have rapidly acquired resistance (Cao LX *et al.*, 2004). Moreover, it has been suggested that they serve as sources of novel bioactive products (Zin *et al.*, 2007; Castillo *et al.*, 2005, 2006, 2007; Ezra *et al.*, 2004; Ryan *et al.*, 2008). It now appears that endophytes are relatively untapped sources of novel natural products, and *Streptomyces* also exist as endophytes within the living tissues of higher plants (Ayuso *et al.*,

2005; González *et al.* 2005). Actinomycetes producing antibiotics are most significant in cancer chemotherapeutic analogs & entities such as, anthracycline, bleomycin, actinomycin, mitomycin and aureolic acid families (Da Rocha *et al.*, 2001; Kwon *et al.*, 2006; Salas and Me'ndez, 2007).

The phylogenetic diversity of isolates was assessed using 16S r RNA analysis. We hope to bioprospect endophytic actinomycetes resources possessing potential to be applied in pharmacy and agriculture field. The present study involved the isolation of endophytic actinomycetes from parts of the cabbage (*Brassica oleracea* or variants), tomatoes (*S. lycopersicum*) and *zea mays*, examining their antimicrobial activities, identify the secondary metabolites, 16S rRNA sequencing, phylogenetic tree, detection of NRPS genes and the antitumour activity.

2. MATERIALS AND METHODS

Sample collection

Healthy leaf, shoot and root tissues of cabbage (*Brassica oleracea*), tomato (*Solanum lycopersicum*) and maize (*Zea mays*) were collected from the plantations in and around Coimbatore district for the isolation of endophytic actinomycetes. The samples were collected carefully from each site to ensure that maximal amount of leaf, shoot and root materials. Samples were placed in plastic bags and taken in an ice-box within 24 hours of collection. All the media and glass wares used were sterilized at 121°C at 15 lb pressure for 20 min.

Segregation of actinomycetes from bio materials

Healthy leaf, shoot and root samples were cut into small pieces (2x2 cm) and washed by running tap water for 1-2 minutes to remove the soil particles completely sterilized by the protocol of Sheng *et al.* (2009). The plant materials were subjected to 4 to 10-min wash in 5% Sodium hypochlorite, followed by a 10-min wash. After the sterility check, the surface sterilized tissues were subjected to continuous drying at 100°C for 15 min. These fragments were mashed with mortar and pestel. An aliquot of 0.1ml of leaves, stems, and roots suspension in phosphate buffer was spread plated on to ISP2, Starch casein agar, Actinomycetes isolation agar plates containing cycloheximide, nystatin, novobiosin, nalidixic acid and rifampicin was mixed with the media (each at concentration of 50µg/ml of medium) and incubated at 28 °C for 7 days.

Detection of Actinomycetes

Colonies on the plates were examined using a microscope and picked on the basis of morphological features and colours of pigmentation. Endophytic actinomycetes were identified using morphological and biochemical criteria.

Morphological characterization

Endophytic actinomycetes isolates were incubated on yeast, malt, Glucose agar, actinomycete isolation agar and starch casein agar with cycloheximide, nystatin, novobiosin, nalidixic acid and rifampicin (each at concentration of 50µg/ml of medium) and incubated at 30°C for 7 days. The strain was carried out for gram staining, shape, size, under light microscope.

Biochemical characterization (Holt 1989):

Various biochemical tests were performed for the identification of various endophytic actinomycetes isolates. The mycelium structure, color and arrangement of spore on the mycelium were examined under oil immersion. Different biochemical tests like starch hydrolysis, triple sugar iron, citrate utilization test, indole test, methyl red test, voges proskauer test, catalase test and oxidase test were performed to characterize actinomycetes.

Screening of Actinomycetes for antimicrobial activity

Primary screening

A modified cross-streak method was used for antimicrobial activity (Lakshmana perumalsamy 1978). Single streak of endophytic actinomycetes was made on surface of the modified nutrient agar and incubated at 28°C (Balagurunathan, 1992). After observing a good ribbon like growth of the endophytic actinomycetes on the plates, the overnight pathogenic bacterial strains, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *E-coli*, *Salmonella* isolated from hospital samples were streaked at right angles to the original streak of endophytic actinomycetes and incubated at 28°C and the incubation distance was measured after 24-48 hrs. Out of 40 isolates 4 strains exhibiting high antimicrobial activity were selected for further studies.

Production of antimicrobial antibiotics (Baiq Erna Listiana)

One plug of 4 endophytic actinomycetes from ISP2 agar culture was transferred into 50 mL of inoculum culture and cultivated on a platform shaker incubator at 27°C and 150 rpm. Production was evaluated in 1 litre of fermentation media. Cultivation was carried out for 14 days on the shaker incubator at 27°C and 150 rpm. Endophytic actinomycetes cells were separated from the supernatant by centrifugation. The supernatant were mixed with equal amount of ethyl acetate to extract compounds and was concentrated at 80° to 90°C.

Separation of antimicrobial metabolites:

The ethyl acetate phase which contains antibiotic was separated from the aqueous phase. It was evaporated to dryness in water bath at 80°-90°C. The obtained compound was used to determine antimicrobial activity.

Amplification and sequencing of the 16S rRNA gene (Jeffrey Janso., 2010)

The endophytic actinomycete-strain 34 was subcultured onto Yeast Malt extract agar slant and was sent to Acme ProGene Biotech, Salem for identification by 16s rRNA sequencing. The preparation of genomic DNA was conducted Sambrook *et al.*, 1989. The 16-S r-RNA-gene was PCR improved using the primers 8FPL (5'-AGAGTTGATC CTGGCTCAG-3') and 1492RPL (5'-GGTTACCTTGTACGACTT-3') (Reyensbach *et al.*, 1994). The BLASTN programme was employed to assess the degree of DNA similarity against those in GenBank database.

Phylogenetic analysis

The phylogenetic analysis sequences were aligned with those of reference strains. Distance analyses were conducted with TREECON 1.3b (Van de Peer, *et al.*, 1994).

Thin Layer Chromatography:

Optimization of mobile phase (methanol:chloroform,70:30) for known antibiotics and test antibiotics was done by using 7.6 x2.4 cm silica gel plates which were prepared and activated at 110 °C half an hour before. Spots on the plates were visualized in an iodine chamber (Busti *et al.*, 2006, Thangadurai *et al.*, 2002).

Column chromatography (Thongchai Taechowisan *et al.*, 2003).**Preparation of column**

In to a column a small amount of glass wool is pushed down to the bottom as a bed. 50g adsorbent (silica) previously activated is taken in a beaker and a small amount of chloroform and methanol was added to make slurry (50 X 7.5 cm). This slurry was carefully poured into the column to avoid air bubbles; the adsorbent was never to be allowed to dry. 30 fractions were collected every 20 minutes. They were run on TLC. This yielded active compound. It was analysed by HPLC. NMR and mass-spectral data were utilized to recognize the series of active analogs.

HPLC Analysis (Baiq Erna Listiana)

The HPLC was used to identify the active compound from active fraction. The HPLC system used an elution solvent with a ratio of water: acetonitrile (8:2). The system was run for 60 minutes with a flow rate of 1 ml/min. 20µL of sample was injected in to the column. The separation was achieved using Column ID 4.6, length 250 mm. The compounds were detected by 215 nm with a UV detector. It was carried out at SITRA.

NMR studies of Structure elucidation of the compounds (Thongchai Taechowisan *et al.*, 2003).

Optical rotations were measured on a Perkin-Elmer 241 polarimeter, IR spectra on a Perkin-Elmer 1 spectrometer, 1H of NMR spectra on a Bruker DRX 500 spectrometer, and EI-MS and GC-MS respectively on a Hewlett-Packard 5989 B and a Finnigan/Thermo Quest Mat 95 XL mass spectrometer in IIT, Chennai.

PCR amplifications with biosynthetic genes primers (Jeffrey Janso *et al.*, 2010)

NRPS gene (Nonribosomal peptide synthetase) codes for production of secondary metabolites. The domains were augmented with the degenerate primer pair A3F(5'-ACSTCGGCWCSACCGGCCIGCCSAAG-3')/ A8R(5'AGCTSAYSCGSWRGCCSCGSAYCTTSACCTG-3') (Haltli *et al.*). The GeneRuler DNA ladder mix was used to size the bands from the PCR products.

Antitumor activities of endophytic actinomycetales metabolites on human cell lines**Cell line**

The human breast cancer cell line (MCF-7) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS).

Cell management practice

The cells were initially dissolved in neat dimethylsulfoxide (DMSO) and an aliquot of the sample to make the final sample concentrations.

MTT test

The percentage cell viability was then calculated with respect to control as follows

$$\% \text{ Cell viability} = [\text{A}] \text{ Test} / [\text{A}] \text{ control} \times 100$$

3. RESULT

The present study was undertaken using three plants namely tomato (*Solanum lycopersicum*), cabbage (*Brassica oleracea*) and maize (*Zea mays*). A total of 45 endophytic actinomycetes were isolated. 4 strains which showed optimum antagonistic property were selected for further studies. The cultural characterization of selected endophytic actinomycete was studied by using different media.

Plate.1. Cultural Characterization of isolates**Plate.2. Primary Screening of actinomycetes**

All the 40 isolates were subjected to primary screening. The strain which showed high antagonistic activity was used for further research.

Biochemical characterization of endophytic actinomycetes

Biochemical test were performed like Indole, MR, VP, Citrate, Catalase, Oxidase, and the result were tabulated (Table 1), (Holt J.G, 1989). The outcome exposed that the complete isolated strains obtained from three plants belonged to *Streptomyces* sp, *Micromonospora* sp and *Nocardia* sp. (Table 1).

Table 1: Outcome from biochemical analysis

S.NO	Biochemical characterization	Strain7	Strain 15	Strain 22	Strain 34
1	Gram staining	Gram Positive rods	Gram Positive rods	Gram Positive rods	Gram Positive rods
2	Type of Spore	Long chain spore	Spiral chain	Long chain spore	Filamentous
3	Indole	-	-	-	-
4	Methyl red	-	+	-	-
5	Vogues proskauer	-	-	-	-
6	Citrate	+	+	-	+
7	Nitrate	-	-	-	-
8	Urease	+	-	-	+
9	Triple sugar iron test	Alkaline slant	Alkaline /alkaline bud	Alkaline slant	Alkaline slant
10	Oxidase	+	+	+	+
11	Catalase	+	+	+	+
12	Starch	+	+	+	+

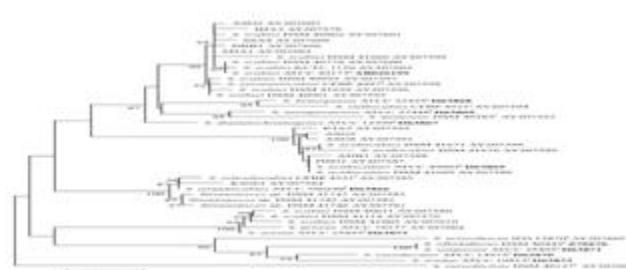
+ Positive - Negative

Sequence alignment of Phylogenetic analysis

S34 was subsequently identified using partial 16S rRNA sequencing and phylogenetic analysis. The results showed that the isolate S34 was *Streptomyces sampsonii*. Phylogenetic tree analysis of the endophytic strain with some closet members clearly indicated that they were dissimilar and belonged to different genus/species.

Sequence alignment of Strain 34-*Streptomyces* sp

GenBank KF511957 (Phylogenetic analysis of *Streptomyces sampsonii* species)



Thin Layer Chromatography

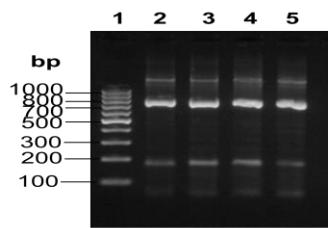
When visualized under iodine vapour each extract showed four spots. The retention factor of the moved spot is given in the (Table2). It indicates the production of antibiotic compound.

Table 2: Results for TLC

R _f values				
Isolates	S 7	S 15	S 22	S 34
R _f values	0.96	0.98	0.96	0.91

Plate.3**HPLC**

The HPLC-UV chromatogram of the isolate exhibited most exciting diversity of the chemical constituents. Strain 34 exhibited three different peaks at retention time (R_t) 1.905, 2.608 and 3.027.

**Figure 1: HPLC****PCR Product**

Agarose Gel (1.5%) showing 100bp DNA ladder and PCR product

- Lane 1 – 100bp DNA Ladder
- Lane 2 – PCR product of S1 (750bp)
- Lane 3 – PCR product of S2 (750bp)
- Lane 4 – PCR product of S3 (750bp)
- Lane 5 – PCR product of S4 (750bp)

Figure 2: Biosynthetic gene primers (NRPS)

Presence of biosynthetic gene NRPS was confirmed in all 4 isolates.

NMR -STRUCTURAL IDENTIFICATION OF ANTIBIOTIC

In an attempt to establish the chemical structure of an antibiotic produced by strain S 34, spectral studies such as UV, IR and NMR were performed. The λ max of the isolated compound by UV analysis was 216 nm. The basic data obtained by NMR spectroscopical study and their corresponding groups are given.

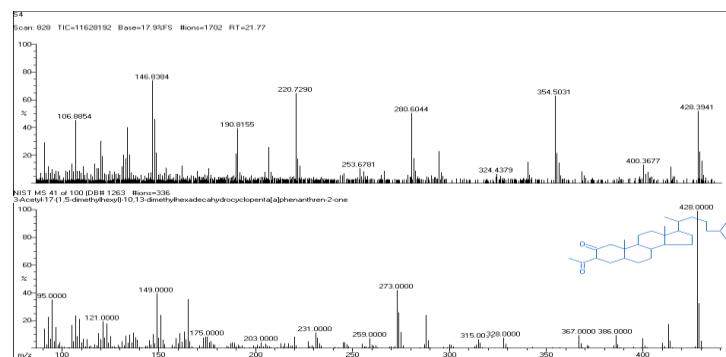


Figure 3: GC of Strain 15

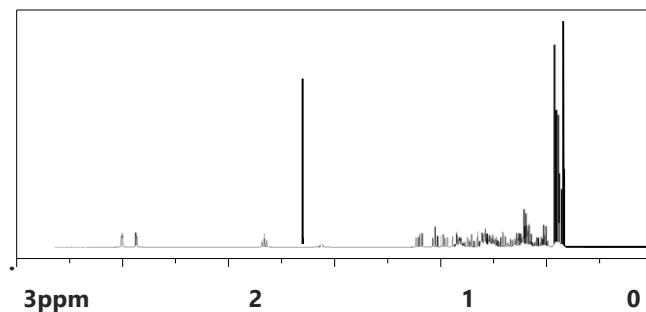


Figure 4: NMR of Strain 15

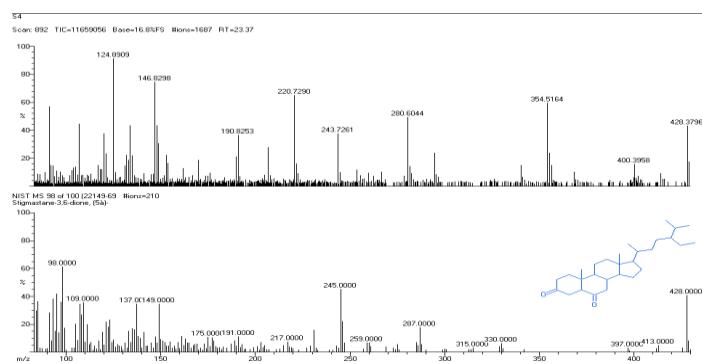


Fig.5. GC of Strain 34

GC MS and NMR depicts the production of 3 acetyl 17-1,5, methyl hexyl, 10,13, dimethyl hexa deca hydro cyclopenta phenenthren 2- one by strain 15 and stigmastane 3,6 dione by strain 34.

Antitumour activity

Organic extracts from strain 15 of the 4 endophytic actinomycetes were effective in causing more than 40% EAC cell death at 160 μ g/ml concentration. There was a dose-dependent relationship of increased number of dead cells with increasing concentration of each metabolite. Investigation of tumor selectivity of the 4 endophytic actinomycete metabolites against selected Breast cancer cell lines indicated effective and promising differential cytotoxicity. S15 gave inhibitory effect of R^2 value of 0.9802.

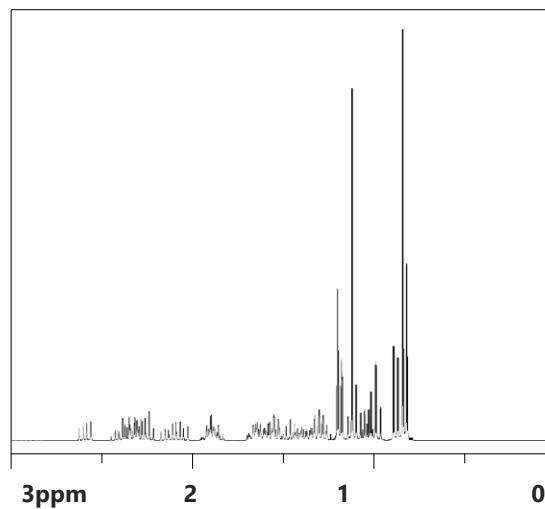
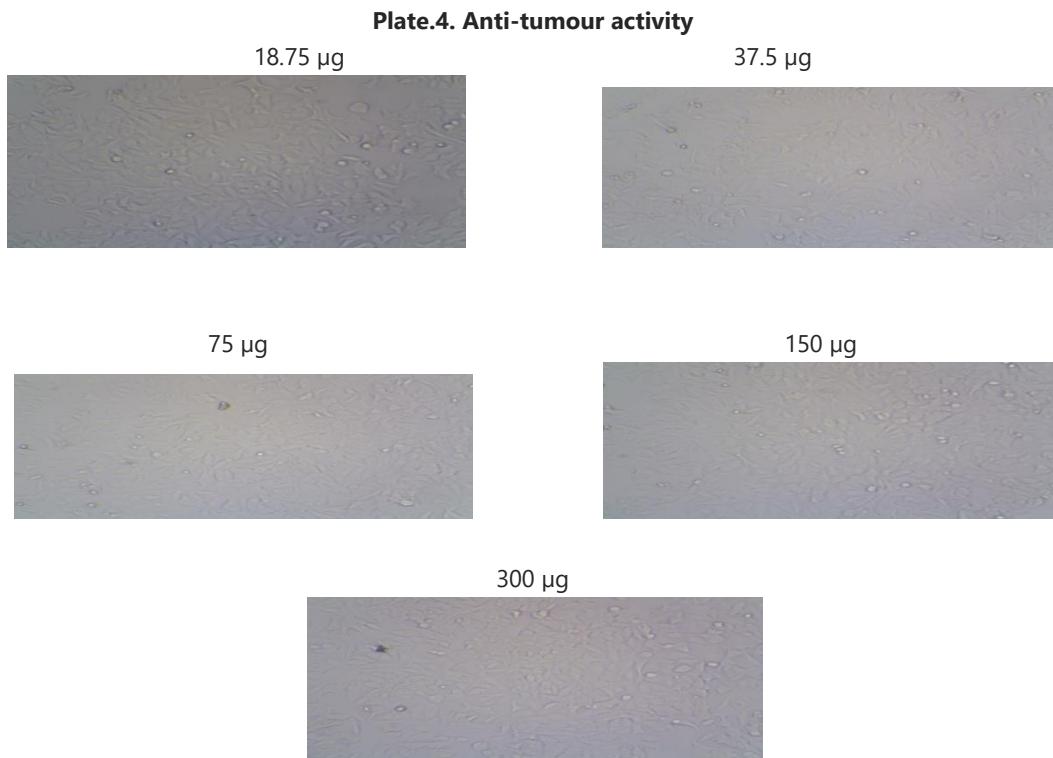


Figure 6: NMR of Strain 34

Strain 15:

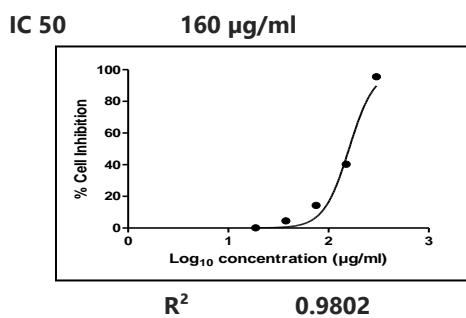
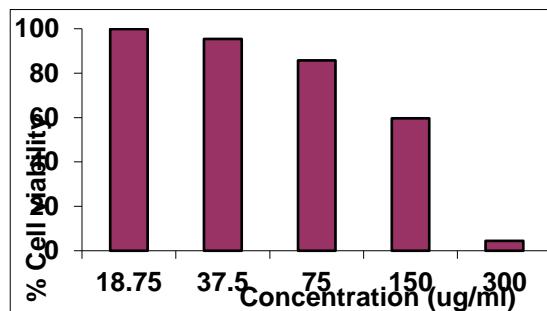
Strain 15 showed high antitumour activity than the other endophytic actinomycete isolates.



Conc	18.75 μg	37.5 μg	75 μg	150 μg	300 μg	Cont
ABS	0.298	0.294	0.269	0.186	0.012	0.305
	0.312	0.288	0.258	0.188	0.012	0.307
	0.31	0.297	0.262	0.176	0.017	0.309
Avg	0.306667	0.293	0.263	0.183333	0.013667	0.307

Conc ($\mu\text{g/ml}$)	% Cell Inhibition
18.75	0.108578
37.5	4.560261
75	14.33225
150	40.2823
300	95.54832

Conc ($\mu\text{g/ml}$)	% Cell viability
18.75	99.89142
37.5	95.43974
75	85.66775
150	59.7177
300	4.451683



Peer-review

External peer-review was done through double-blind method.

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This study has not received any external funding.

Conflict of Interest

The authors declare that there are no conflicts of interests.

Data and materials availability

All data associated with this study are present in the paper.

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